

Comparative Analysis of Antioxidant and Antimicrobial Properties of Raw Theobroma Cacao Beans and Processed Cocoa Extracts

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Abstract Theobroma cacao, renowned for its rich flavor and therapeutic potential, undergoes transformative processes from raw beans to familiar cocoa products that are widely consumed worldwide. This study delves into the comparative analysis of the antioxidant and antimicrobial properties of raw Theobroma cacao beans and their processed counterparts. Using various extraction methodologies, we meticulously quantified and evaluated the phenolic content, antioxidant potential, and antimicrobial efficacy against prevalent microbial strains of *Staphylococcus aureus*, *Escherichia coli*, and *Pseudomonas aeruginosa*. Our findings revealed striking differences in the bioactive compound composition and subsequent biological activities of raw cacao beans and processed cocoa extracts. Processed cocoa extracts exhibited robust antioxidant capacity and antimicrobial properties, which were attributed to their refined nature. The processing stages involved in cocoa production seemingly augment the bioactive compound profile, leading to enhanced antioxidant potency and antimicrobial activity. The IC_{50} values of the processed cocoa powder extracts (PC1 and PC2) were $18\mu\text{g/ml}$ and $26\mu\text{g/ml}$. The IC_{50} of the raw cocoa powder extract was $36\mu\text{g/ml}$ and ascorbic acid, a known antioxidant, had an IC_{50} of $1.5\mu\text{g/ml}$. Understanding the superiority of processed cocoa extracts offers invaluable insights into optimizing processing methodologies to maximize the retention or augmentation of these health-promoting properties. Our findings contribute significantly to the discourse on harnessing the full potential of Theobroma cacao, advocating for informed decisions in cocoa processing to ensure the delivery of products rich in both flavor and holistic well-being benefits.

Keywords: antioxidant, antimicrobial, biofilm, therapeutic, incubation, scavenging, bioactive compounds

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1. Introduction

The Cacao tree, also known as the cocoa tree, is native to the Amazon rainforest. It was first domesticated 5,300 years ago in equatorial South America, before being introduced in Central America by Olmecs (Mexico) [1]. It grows in the foothills of the Andes in the Amazon and Orinoco basins in South America, Colombia, and Venezuela. Cacao trees grow in a limited geographical zone of approximately 20° to the north and south of the equator. Nearly 70% of the world's crops are grown in West Africa [2]. The major cocoa bean-growing countries in the world are the Ivory Coast, Ghana, Indonesia, Cameroon, Nigeria, Brazil, Ecuador, Dominican Republic, and Malaysia, contributing to almost 90% of world production [3,4].



Figure 1. Cocoa pods showing seeds embedded in pulp and dry seeds

The cacao plant was first given its botanical name by Swedish natural scientist Carl Linnaeus in his original classification of the plant kingdom, where he called it *Theobroma cacao* (meaning "food for the gods"). Central to its acclaim are its antioxidant and antimicrobial properties, which are attributed to a rich repertoire of bioactive compounds [5]. As the world's appetite for chocolate continues to grow, it is important to understand

how the processing of cacao beans into cocoa affects its nutritional and therapeutic attributes [1,6].

Cocoa, the end product of *Theobroma cacao* beans, undergoes a series of complex processing steps from harvest to consumption. These steps, which include fermentation, drying, roasting, and grinding, are integral to the development of the characteristic flavor and texture of cocoa products [7,8]. However, they also have the potential to alter the chemical composition and bioactivity of cacao bean constituents, thus influencing their health-promoting properties [9]. The aim of this study was to unravel the nuanced differences in the antioxidant and antimicrobial activities of extracts obtained from raw *Theobroma cacao* beans and processed cocoa. Cocoa contains polyphenols and is a source of many antioxidants [10]. Cocoa contains a variety of chemicals, including flavonoids, which help relax blood vessels. This also helps to lower blood pressure, reduce inflammation, and prevent blockage of blood vessels, and is used to treat diseases with high blood pressure [11,12]. Antioxidants play a major role in preventing lipid oxidation by inhibiting the initiation or propagation of oxidizing chain reactions and are involved in scavenging free radicals [13].

However, the transition from raw cacao beans to processed cocoa involves a transformation that may affect the bioavailability and concentration of these bioactive compounds. Fermentation, a crucial step in cocoa processing, initiates biochemical reactions that contribute to the development of cocoa flavor precursors while potentially altering the composition of phenolic compounds [14]. Understanding the implications of these processing steps on the antioxidant and antimicrobial properties of cocoa is essential for several reasons [15].

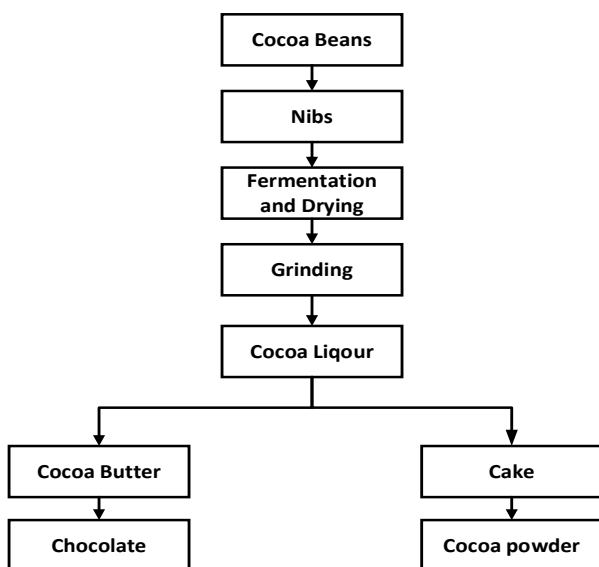


Figure 2. Cocoa bean processing stage [16]

Through meticulous experimentation and analysis, this study aimed to quantify and compare the antioxidant capacities and antimicrobial efficacies of extracts derived from raw cacao beans and processed cocoa. Utilizing state-of-the-art extraction techniques and analytical assays, we sought to elucidate the influence of processing on the concentration and bioactivity of key bioactive compounds in cocoa. By bridging the gap between traditional knowledge and modern science, we aspire to unlock the

full therapeutic potential of *Theobroma cacao*, ensuring its legacy as a truly divine gift to humanity.

2. Materials and Methods

2.1. Materials

Cocoa beans, cast iron pan, spatula, burner, blender, test tubes, test tube rack, measuring cylinder, volumetric flask, beaker, stirrer, UV spectrophotometer, 96-microtitre plate, incubator, crystal violet, ascorbic acid, tannic acid, Muller Hinton broth, microplate reader, DPPH, methanol, cotton, 0.6M sulphuric acid, 28mM disodium hydrogen orthophosphate anhydrous, 4mM ammonium molybdate, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Escherichia coli*, 95% ethanol, micropipette, micropipette tips, micropipette tip holder, 2 processed cocoa powder (labelled as PC1 and PC2) were obtained from the Ashaiman market in Accra, Ghana.

2.2. Methods

2.2.1. Preparation of Raw Cocoa Powder Extract

The dry cocoa seeds obtained were allowed to ferment for five days. The fermented beans were placed in the sun for several days. After drying, the beans were roasted in a cast iron pan for five minutes under high heat and left for an additional 20 min under low heat until the husks became brittle. The nibs were then separated from the husks and ground in a dry blender until a fine powder was obtained.



Figure 3. (a) Dry cocoa beans (b) Grounded cocoa beans

2.2.2. 2,2-Diphenyl-1-Picrylhydrazyl (DPPH) Free Radical Scavenging Activity

Concentrations of 2.5µg/ml, 5µg/ml, and 10µg/ml of raw cocoa powder extract, and the processed cocoa extracts were prepared by dissolving various quantities in methanol. Ascorbic acid was used as the standard antioxidant and was prepared in methanol at the same concentrations as those of the cocoa powder extracts. DPPH solution (0.0002nM concentration of DPPH was prepared in methanol. DPPH solution (3 ml of DPPH solution was mixed with 1 ml of the standard solution in test tubes at room temperature (25 °C). The mixture was then incubated in the dark for 30 min. Absorbance was measured using a UV spectrometer at a wavelength of 517 nm. This process was repeated for all the three extracts. The % DPPH scavenging activity was calculated using the following equation [8,18]:

$$= \left[\frac{A_{\text{control}} - A_{\text{test}}}{A_{\text{control}}} \right] \times 100$$

Where;

- A_{control} is the absorbance of the control (DPPH in methanol without test sample).
- A_{test} is the absorbance of the test sample (DPPH in methanol with extract).

A linear regression analysis was carried out to calculate the inhibitory concentration of the sample required to scavenge 50% of DPPH radicals [18].

2.2.3. Antioxidants

Antioxidants are microconstituents present in diet that can delay or inhibit lipid oxidation by inhibiting the initiation or propagation of oxidizing chain reactions and are involved in scavenging free radicals [8]. Free radicals are reactive oxygen species that include all highly reactive oxygen-containing molecules. Flavonoids act as antioxidants by directly neutralizing free radicals and chelating metals (Fe^+ and Cu^+) that enhance highly aggressive reactive oxygen species, inhibit enzymes responsible for reactive oxygen species production (xanthine oxidase), and protect antioxidant defenses [13]. Antioxidants lower the risk of chronic diseases such as heart disease, cancer, and neurodegenerative disorders. Antioxidants also support immune functions, enhance skin health, and slow aging. Their presence in food promotes overall health and well-being, making them essential for a balanced diet [19].

2.2.4. Determination of Total Antioxidant Capacity

A standard reagent solution was prepared by adding 50 ml equal volumes of 0.6M sulphuric acid, 28mM disodium hydrogen orthophosphate anhydrous, and 4mM ammonium molybdate. Ascorbic acid was used as a positive control, and concentrations of 0.039µg/ml to 10µg/ml were prepared in methanol. Three milliliters of the standard reagent solution was added to 1 ml of each extract at various concentrations. The mixture was then incubated at 95°C for 90 min. The mixture was allowed to cool, and absorbance was measured at a wavelength of 695 nm. Methanol was used as blank. All three extracts were subjected to the same procedure [5,8,20].

2.2.5. Determination Of Total Phenolic Content

Concentrations of 2.5µg/ml, 5µg/ml, and 10µg/ml of raw cocoa powder extract, and the processed cocoa extracts were prepared by dissolving various quantities in methanol. Tannic acid was used as a control. A volume of 0.5 ml of the 0.1 ml of 0.5N folin-ciocalteu reagent and the mixture was incubated at room temperature for 15 min. A volume of 0.2% w/v saturated sodium carbonate (2.5 mL) was added to the mixture and incubation was continued for 30 min at room temperature. Absorbance of the mixture was measured at a wavelength of 760 nm. Methanol was used as blank. This procedure was performed for all three extracts [8,18,20].

2.3. Evaluation Of Anti-Biofilm Activity

Biofilm formation is a sophisticated survival strategy

employed by bacteria, which allows them to adhere to surfaces and thrive in diverse environments. It begins with the reversible attachment of planktonic (free-floating) bacterial cells to a surface, facilitated by weak physicochemical interactions such as van der Waals forces and electrostatic interactions [21]. Upon attachment, bacteria produce extracellular polymeric substances (EPS), which form a protective matrix encasing bacterial cells [22].

As the biofilm matures, bacterial cells within the EPS matrix undergo physiological changes, transitioning into sessile or non-motile states. This transition triggers the expression of genes involved in biofilm formation, including those encoding adhesins, exopolysaccharides, and quorum-sensing molecules. Quorum sensing enables bacteria to communicate with one another and coordinate collective behaviors such as EPS production and biofilm development. Biofilm architecture becomes increasingly complex as bacterial cells continue to proliferate and secrete EPS [23]. This three-dimensional structure provides protection against environmental stressors, including antimicrobial agents, and hosts immune responses, making biofilm-associated infections difficult to treat.

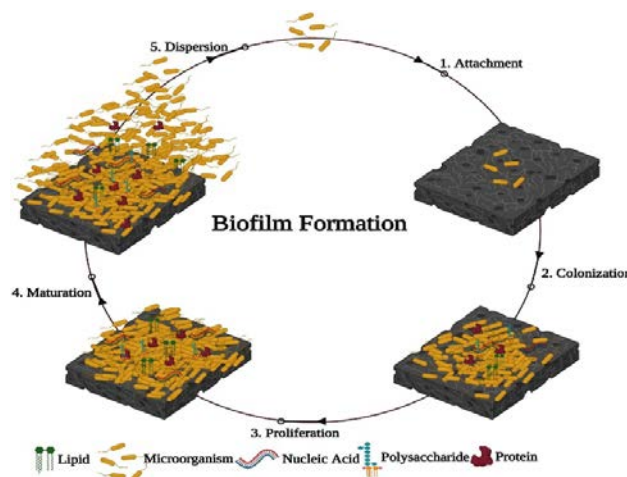


Figure 4. Developmental stages of bacterial biofilm formation [26]

Ultimately, biofilm formation facilitates bacterial persistence and enhances resistance to adverse conditions, posing significant challenges in various fields including healthcare, food safety, and environmental management. Understanding the mechanisms underlying biofilm formation is crucial for developing effective strategies for preventing and combating biofilm-associated infections. Biofilm formation begins when free-floating microorganisms such as bacteria are exposed to an appropriate surface. Generally, biofilm formation by bacteria pathogens on any substratum or layer involves five major stages [24,25].

- **Attachment.** At the initial stage, free-swimming planktonic cells reversibly attach to biotic or abiotic surfaces through weak acid-base, hydrophobic, van der Waals, and electrostatic interactions.
- **Colonization.** Bacterial pathogens irreversibly attach to surfaces through stronger interactions, such as collagen-binding adhesive proteins, lipopolysaccharides, flagella, and pili.
- **Proliferation.** Multilayered bacterial cells are profoundly accumulated, and enormous amounts of EPS are produced.

- **Maturation.** The attached multilayered bacterial cells grow into mature biofilms with a typical 3D biofilm structure.
- **Dispersion.** After complete development of the biofilm, it is disassembled or dispersed using mechanical and active processes.

2.3.1. Inhibition of Biofilm Formation (Inhibition of Initial Cell Attachment)

Fresh microbial cultures were prepared in Muller Hinton broth for *E. coli*, *P. aeruginosa* and *Staph. Aureus*, and incubated for 24 hours at 37°C. Muller Hinton broth was prepared and sterilized at 121°C. 100µg/ml, 50µg/ml and 25µg/ml concentrations of the extracts were prepared. The cultures were diluted with sterile water until a culture count of 0.5, a MacFarland standard, was obtained. 100µl of the broth were added to an individualized flat-bottom 96-well microtiter plate. 100µl of the standardized concentrations of the microbial cultures were added to individual 96-well microtiter plates and incubated at 37°C for 4 h without shaking. The plates were later removed and 100µl of the extract concentrations used for each organism varied since it corresponded to a percentage of its MIC value. Extract concentrations of 100%, 50%, and 25% MIC for each of the test organisms were used for the determination. A mixture of broth and extract served as a negative control. The biomass was quantified using a modified crystal violet staining assay. This is a modification of the method described previously [27,28].

2.3.2. Determination of Specific Biofilm Formation by Crystal Violet Staining

After a 24 hours incubation period, the microtiter plates were removed, and each well was washed thoroughly with sterile water to remove free-floating cells. The plates were air-dried at room temperature for 30 min. The wells were stained with a 0.1% aqueous solution of crystal violet for 15 min and then incubated at 37°C for 30 min. The stain was removed after incubation, and the excess stain was washed with sterile water three times. The dye-bound cells on the sides of the wells were solubilized by adding 125µl of 95% ethanol solution to each well. The mixture was incubated for 15 min at 37°C. Absorbance was measured after incubation at 570 nm using a microplate reader [28]. Specific Biofilm formation (SBF) was calculated as:

$$\left[\frac{OD_e - OD_c}{G} \right]$$

Where;

- OD_e is the optical density of the attached and stained bacteria measured at 570 nm.
- OD_c is the optical density of the control wells containing sterile water at 570 nm.
- G is the optical density of the cell growth in the broth at 630 nm.

2.3.3. Destruction of Pre-Formed Biofilms (Assessment of Destruction of Biofilm Mass)

A standardized fresh microbial culture of each organism was prepared by inoculation and culturing in Muller-Hinton broth at 37°C for 24 h. After incubation, the microbial cultures were reconstituted with sterile water

to obtain a MacFarland standard of 0.5. A volume of 100µl of the organism suspension was added to a 96-welled microtiter plate containing 100µl of broth and incubated for 48 h at 37°C without agitation. A volume of 100µl of the extract was then added. The plates were then incubated at 37°C for 24 h. Broth containing the extract concentrations served as the negative control. The biomass of was then quantified using a modified crystal violet staining assay [27].

2.3.4. Determination of % Biofilm Inhibition by Crystal Violet Staining

After 24 h of incubation, the microtiter plates were removed, and each well was washed thoroughly with sterile water to remove free-floating cells. The plates were air-dried at room temperature for 30 min and further dried at 60 °C in an oven for 45 min. The wells were stained with a 1% aqueous solution of crystal violet. The stained walls were incubated at 25 °C for 15 min. After incubation, the wells were washed thrice with sterile water and allowed to air dry for 30 min, after which 125µl of 95% ethanol solution was added to each well to solubilize the cells. 100µl of the solubilized mixture from each well were transferred into a new 96-welled microtiter plate, and the absorbance was determined at 590 nm using a microplate reader[28]. The mean absorbance was determined, and the percentage of biofilm inhibition was determined according to the equation below.

$$= \left[\frac{OD \text{ (negative control)} - OD \text{ (test extracts)}}{OD \text{ (negative control)}} \right] \times 100$$

Where;

- The OD (negative control) was the optical density of the negative control.
- The OD (test extract) is the optical density of the test extract.

2.4. Statistical Analysis

The data obtained were analyzed using one-way Analysis of Variance (ANOVA) on a completely randomized design using Excel Data Spreadsheet. Statistical significance was set at $P < 0.05$.

3. Results and Discussion

DPPH free radical scavenging is an accepted mechanism for screening the antioxidant activities of plant extracts. The effect of antioxidants on DPPH is due to their hydrogen-donating abilities [29]. In the DPPH assay, the violet DPPH solution was reduced to a yellow product, diphenyl picryl hydrazine, by the addition of the extract in a concentration-dependent manner [30]. This method has been used extensively to predict antioxidant activity because of the relatively short time required for analysis [8]. The IC_{50} values of various extracts were determined (Table 1). Figure 5 shows the DDPH scavenging activities of the raw cocoa powder extracts and processed cocoa extracts compared to ascorbic acid. It was observed that, the processed cocoa extracts (PC1 and PC2) had higher activity than the raw cocoa extract. The total antioxidant

and phenolic capacities of the extracts were also determined. The two processed cocoa powder extracts exhibited greater antioxidant and phenolic capacities than the raw cocoa extract (Figure 6 and Figure 7).

Table 1. IC₅₀ Values of Extracts

Extract	IC ₅₀ Value (µg/ml)
Ascorbic Acid	1.5
Raw Cocoa	36
PC1	26
PC2	18

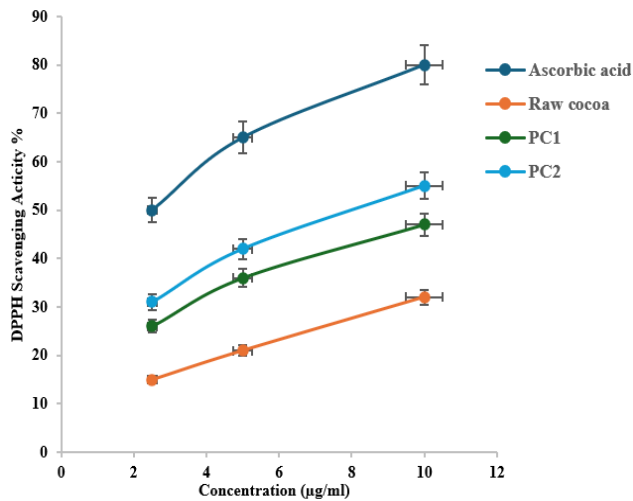


Figure 5. DPPH Scavenging Activity of Extracts

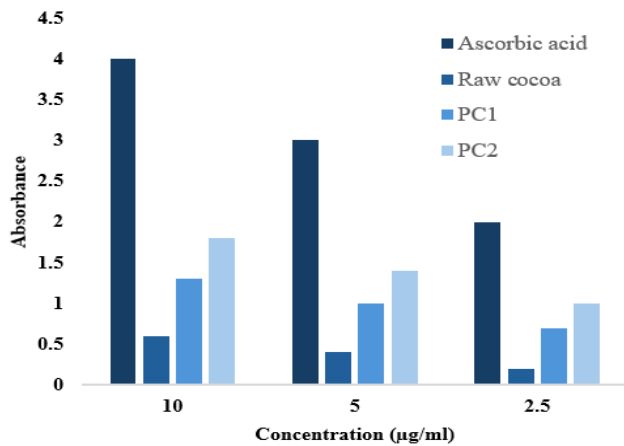


Figure 6. Total Antioxidant Capacity (IAC)

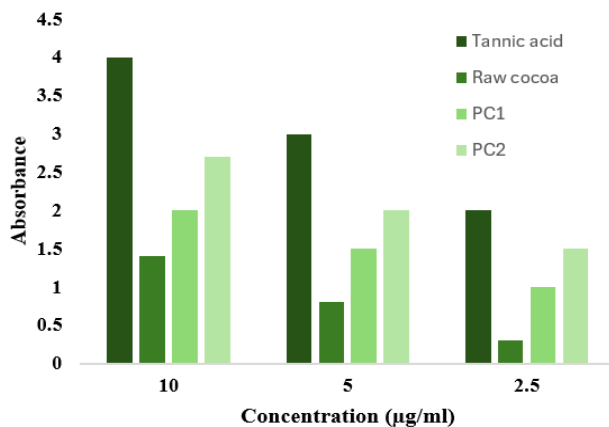


Figure 7. Total Phenolic Capacity of Extracts

As shown in Figure 8 and Figure 9, both the raw and processed cocoa extracts exhibited biofilm inhibition and destruction. Similar to the antioxidant activities of the two samples, the processed cocoa extracts demonstrated the maximum inhibition and destruction of the biofilm, as shown in Figure 8 and Figure 9. The raw cocoa extract showed minimal inhibition of biofilm formation compared with the processed cocoa extracts (Figure 10). The inhibition of biofilm formation can be attributed to the ability of phenolic compounds to disturb the expression of genes in the age system related to quorum sensing, which is vital for biofilm formation [31]. They block quorum sensing inducers, such as acyl homoserine lactones (AHL), auto-inducers, and auto-inducers type 2. This can also be attributed to the fact that the phenolic compounds present also play a significant role in inhibiting bacterial adhesion by interfering with the forces (van der Waals force of attraction, electrostatic attraction, sedimentation, and Brownian movements) that are responsible for the support of bacterial attachment to various surfaces [31,32]. Processed cocoa extracts exhibited maximum inhibition and destruction of biofilms formed by cultured organisms. *Staphylococcus aureus*-containing wells showed maximum biofilm inhibition and disruption compared with wells containing *Escherichia coli* and *Pseudomonas aeruginosa* [31].

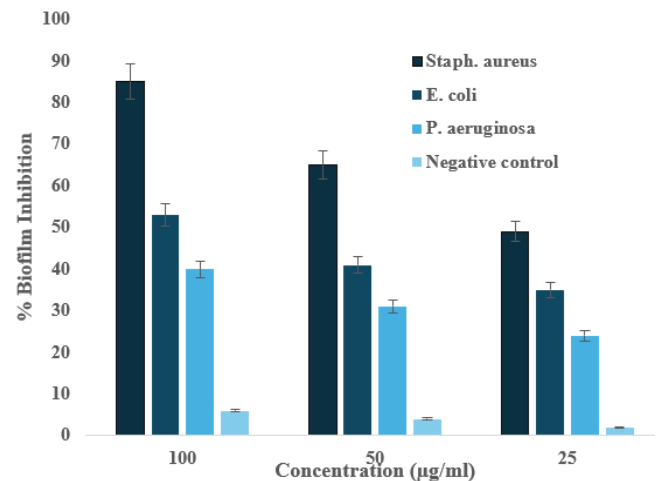


Figure 8. Biofilm Inhibition of PC2 Extract

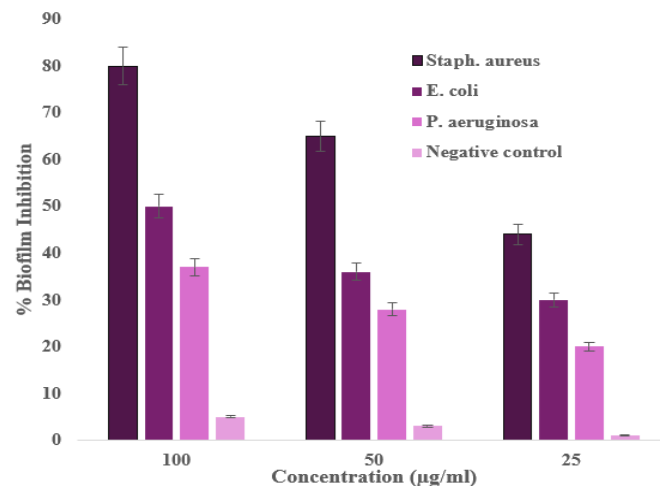


Figure 9. Biofilm Inhibition of PC1 Extract

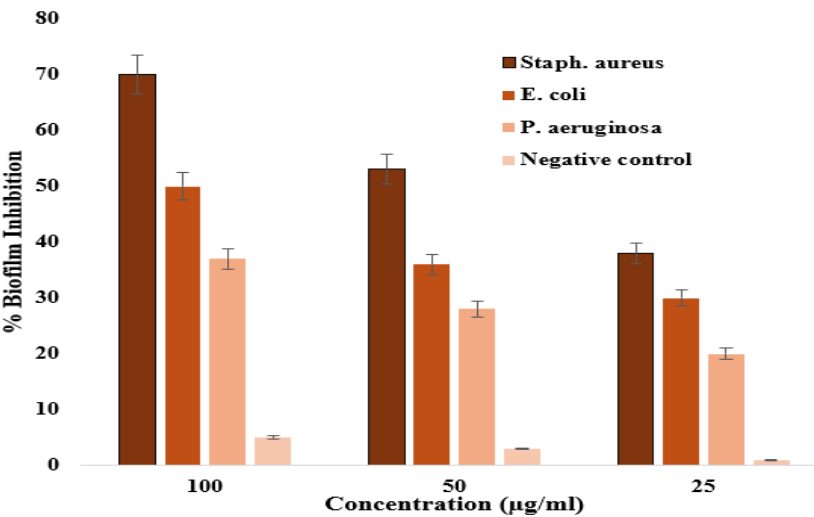


Figure 10. Biofilm Inhibition of Raw Cocoa Extract

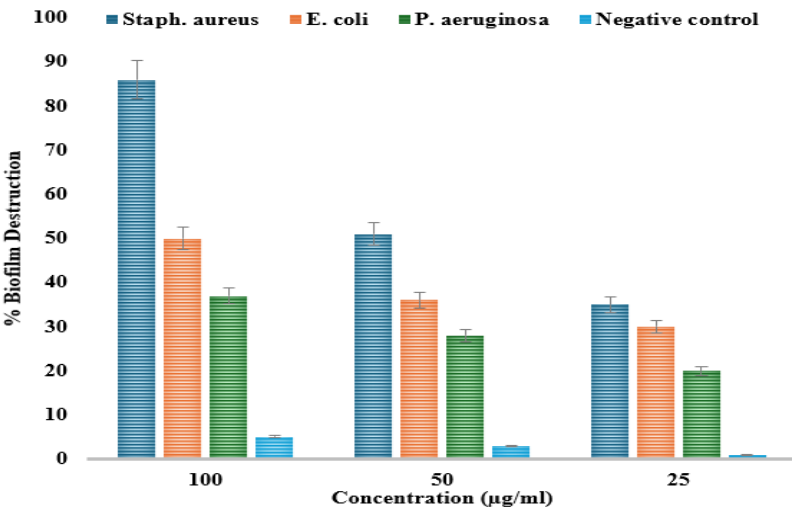


Figure 11. Biofilm Destruction of PC2 Extract

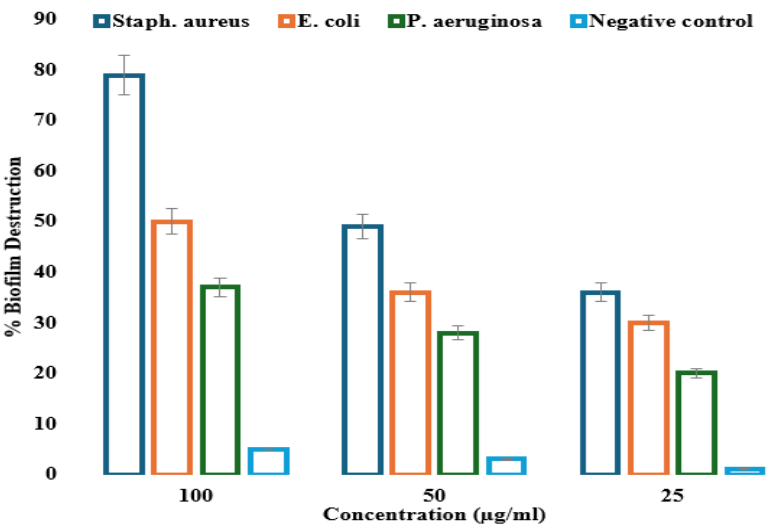


Figure 12. Biofilm Destruction of PC1 Extract

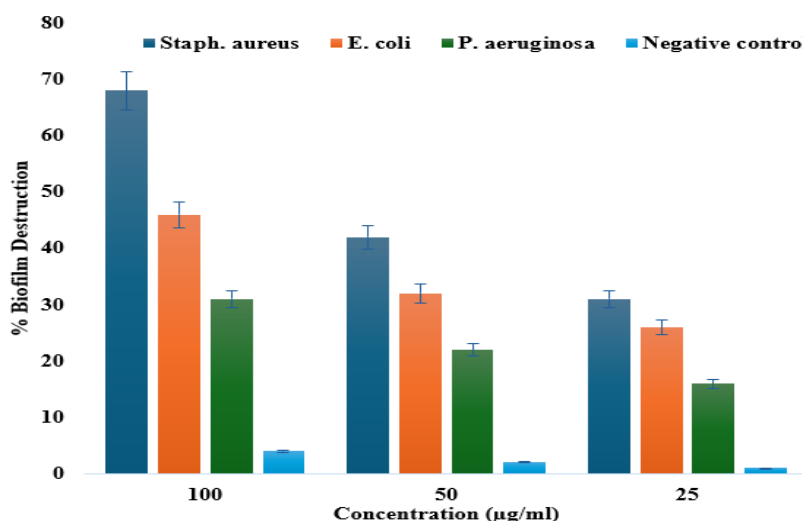


Figure 13. Biofilm Destruction of Raw Cocoa Extract

The exploration of both the antioxidant and biofilm inhibition activities of the cocoa powder extract offers a multifaceted perspective on its potential health-promoting and antimicrobial properties. Our study provides compelling evidence supporting the dual functionality of cocoa powder extract as an antioxidant and biofilm inhibitor, highlighting its versatility in diverse applications [33,34]. The antioxidant activity of the cocoa powder extract, as demonstrated by its ability to scavenge free radicals and inhibit oxidative damage, underscores its potential role in mitigating oxidative stress-related diseases and promoting overall well-being. Polyphenols, particularly flavonoids and procyanidins, are believed to be the primary contributors to the antioxidant properties of cocoa, owing to their ability to neutralize reactive oxygen species and modulate cellular signaling pathways [17]. By harnessing the antioxidant power of cocoa powder extract, we can potentially combat chronic diseases and enhance cellular defense mechanisms against oxidative damage [13].

Simultaneously, the observed biofilm inhibition activities of the cocoa powder extract offer promising prospects for combating microbial infections and controlling biofilm-related complications. Biofilms represent a formidable challenge in various industries, including healthcare, food processing, and environmental management, owing to their resilience to conventional antimicrobial agents and their role in persistent infections and contamination [32]. The ability of cocoa powder extract to disrupt biofilm formation and destabilize the biofilm matrix presents a novel approach to combating biofilm-associated pathogens and preventing biofilm-related complications. The synergistic effects of the cocoa powder extract on antioxidant and biofilm inhibition activities have significant implications for various applications. In the food industry, incorporating cocoa powder extract into food formulations or packaging materials may enhance product stability and safety by preventing oxidation and inhibiting microbial contamination. In healthcare settings, cocoa powder extract can serve as a natural antimicrobial agent for preventing biofilm formation on medical devices or promoting wound healing by combating biofilm-associated infections.

However, further research is needed to elucidate the underlying mechanisms of the antioxidant and biofilm

inhibition activities of the cocoa powder extract and to optimize its efficacy for specific applications. Factors such as extraction methods, formulation, and interactions with other compounds may influence their bioactive properties and overall effectiveness [15].

4. Conclusion

Based on all the elaborate discussions on the findings of this research, it can be concluded that the antioxidant and antimicrobial capacities of the raw cocoa powder extract is lower than that of the processed cocoa powder extracts. This is most likely due to the traditional roasting of beans, which is assumed to reduce the polyphenol content and other phytochemicals of the beans; hence, phenolic activity, such as antioxidant capacity, was reduced [35]. Our findings hold the potential to inform not only consumer choices, but also industry practices, paving the way for the development of cocoa products that deliver both indulgence and health benefits. This study highlights the significant antioxidant and antimicrobial activities of cocoa powder extract. Further research is warranted to elucidate the mechanisms underlying these effects and to explore novel applications of cocoa powder extract in various industries.

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Conflict of Interest

The authors declare that there are no conflicts of interest for publication of this paper.

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